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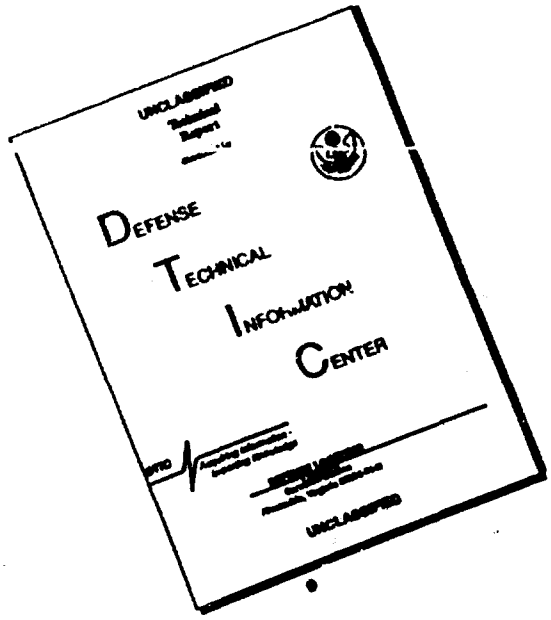

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Membrane Filters and their use in Microbiological Studies of Water,

by
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INTRODUCTION.

Membrane filters (MF) are practically without fault in solving the problem of separating suspensions from various types of liquid media.

Although, as will be indicated later, membrane filters were first manufactured abroad (in Germany), they were first used extensively in microbiology, and especially in studying water in the USSR.

The aim of this short survey is to (1) give a precise description of the membrane filters, (2) examine the various trends marking their use in hydrobiology in general and in water microbiology in particular, (3) to analyze the present-day status of the use of membrane filters in the field in foreign countries and, finally, (4) to point out ways for further work with membrane filters on the basis of personal experience and thus contribute to heightening interest in them which was, unfortunately, grown somewhat less in the USSR recently although the possibilities which they offer investigators have not been exhausted.

MEMBRANE FILTERS.

What we call membrane filters in Germany and the USSR, and

are now called molecular, isoporous or milliporous filters in the US, are made from nitrocellulose. They may also be made from other cellulose esters, as for example acetylcellulose.

Rather detailed information on the composition and properties of membrane filters may be found in Sander and Zikovsky's (1937) and Tovarnitskiy and Glukhareva's (1951) monographs. The first to make membrane filters and study them were Zsigmondy and Bachmann (1918).

At present there are a number of types of membrane filters. The most widely distributed are filters made with alcohol-ether (collodium) or acetone solutions of nitrocellulose as a base. In developing the methodology for a direct microscopic count of bacteria in water (Razumov, 1932) we were forced to make our own membrane filters.

Our first filters were made in 1929 at the VODGEO (All Union Scientific Research Institute for Water Supply, Sewer Systems, Hydraulic Structures and Hydrogeological Engineering) institute with the collaboration of G.I.Dolgov according to Bachmann's simple recommendations.

According to his formula the filters are made from collodium or a solution of movie or photographic film with the emulsion layer removed in acetone. After standing and being carefully filtered these solutions are poured onto a glass in a perfectly horizontal position. After evaporation of the liquid components of the mixture, the remaining filter is cut into disks on the required dimensions. By varying the relative amount of acetone in the first case and the alcohol in the

second, one may obtain filters of different density. The presence of filters and plasticizing agents in the movie and photographic films impelled us to replace them with collodion cotton of specific brands which produced filters of more standard quality.

The first original formula for making membrane filters in Russia was proposed by Klimova and Voroshilova (1932). In this case acetone and ethyl acetate were solvents of nitrocellulose and isoamyl alcohol was the congulating agent. As the solvents evaporate the relative amount of coagulating agent in the solution of the glass increases and this is what determines the micro- and macroscopic structure of the filter.

The production of small-dimension filters is quite easy in the laboratory but it is difficult to make them of standard quality since a number of conditions must be met exactly, in particular: standard quality of the nitrocellulose (collodion cotton) according to the degree of nitration and viscosity of its solutions, solvents and coagulating agents, temperature, atmospheric moisture of the room where the filters are made, the evaporation cycle of the mixture poured out on glass, etc.

In consequence of this, it is preferable to use factory-made filters. The first factory to make membrane filters was set up in Goettingen, Germany, on the basis of Zsigmondy's work; it is still in existence.

In Russia the production of membrane filters on a scale to satisfy the needs of Moscow Water Works laboratories was started in 1932-1933. Later production was increased to meet the requirements of the whole country at the experimental ultrafilter plant of the Ministry of Communal Services RSFSR (in Mytishchi).

The first membrane filter factories were established in the US in 1951-1952.

The structure of the filters is quite complicated. We may distinguish: (1) the almost ideally even and smooth surface turned toward the glass during manufacture and (2) the wavy side turned upward toward the air. Filtration is usually slower if intake is from the less smooth ("air") side than when the liquid passes into the "glass" side. It is supposed that the pores are broader on the "glass" side (cf. LAander and Zakovskiy, 1937, Page 88).

Some types of membrane filters, when examined from the air side or held up to the light, show a more or less clearly pronounced system of polygonal cells (cellular structure). The faces of the cells appear to be more finely pored than the central parts.

Suspensions separated from water on these membrane filters are distributed unevenly in spots. The appearance of this type of structure depends on the composition of the original mixture, in particular on the introduction of ethyl alcohol into it. This structure is completely lacking in filters made according to Dianova and Vorozhilova's

method (1932).

The glass side of membrane filters is usually shiny and enamel-like (velvety in more porous filters) and sometimes the shininess is impermanent and rubs off easily or there is a(film) fine powdery deposit. To insure uniformity in the distribution of the suspended matter (bacteria) on the filter we recommend removing this fine deposit with a bit of absorbent cotton (for the preparation of membrane filters for bacteriological analyses, cf. Drachov, Razumov and others, 1953, page 267). Concepts of the fine microscopic structure of membrane filters were until recently largely schematic and to a great degree hypothetical (a system of tiny channels with round or slot-like cross sections).

Helmcke (1953) made an electron optical study of German membrane filters after powdering them with metal, which permitted him to examine the details of surface structure and the absorption of hydrocarbons with their subsequent evaporation, by dissolving of the body of the filter with acetone in both cases and by stereoscopic examination of the residue - the "imprint" of the pore system.

Five types of filters were studied with a water penetration defined as the speed of filtration of 100 milliliters through an area of 100 square centimeters in from 100 to 1 seconds. The cavities of the densest filters consist of very fine vesicles distributed throughout the body of the membrane filter and of

relatively uniform dimensions. When the water penetrability of the membrane filter increases, the size of the cavities increases; the body of the membrane filter is a profusely branched system of tiny canals. The outlets of the canals on the surfaces make them uneven and irregular. The easily penetrable filters have rather large cavities but the main mass of the filter is spread out between them like little islands. Their surface is still more uneven.

According to calculations made by Goetz and Tsuneishi (1951) the pores make up 75-80 percent of the volume of bacteriological (molecular) membrane filters while the body substance is only 15-20 percent. On a membrane filtration surface 50 millimeters in diameter there are approximately 500 million pores.

Filters made in Russia are disks 35 millimeters in diameter, 0.1 millimeter thick and weighing appreximately 20-30 milligrams. The porosity of the membrane filters is characterized by water penetrability defined as the rate of passage through the filtration area (30 millimeters in diameter) of moist membrane filters of 500 milliliters of distilled water and by penetrability of dry membrane filters by air as expressed in atmospheres. The specifications of membrane filters are presented in the table.

For direct bacteria count there are membrane filters with an 18-millimeter diameter.

SPECIFICATIONS OF MEMBRANE FILTERS(ACCORDING TO ALL FACTORY OF THE MINISTRY OF COMMUNAL SERVICES RSFSR)

Factory No.	Water permeability (average)	Air permeability (atmospheres)	Average pore size (microns)
1	9 min	2 - 2.5	0.3
2	4.5 min	1.8 - 2.0	0.5
3	2.15 min	1.5 - 1.8	0.7
4	65 sec	1.2 - 1.5	0.9
5	30 sec	0.5 - 1.2	1.2
Preliminary	12 sec	-	2 - 5

Filters made in Germany are 0.1 millimeter thick, total diameter 42 millimeters and an average pore diameter in different types of membrane filters of 0.7 - 2.0 microns (Kruse, 1949). Their water permeability has been cited earlier (Helmcke, 1953).

According to Bush (1953) two types of "molecular" membrane filters are manufactured in the US: AA- "aerosol" -- for separating suspended matter and bacteria from gaseous media and HA - "hydrosol" for separating suspended matter and bacteria from loquid media. HA filters are put,out in the form of disks (47 millimeters in diameter working surface of 9.6 square centimeters and thickness of 0.15 millimeters). According to Goetz (1951) the filters are 50 millimeters in diameter. Filtration rate: 100 milliliters of distilled

water at room temperature and one atmosphere pressufe will pass through in 5 seconds, or 15 seconds in the case of denser membrane filters.

The most important criterion for the quality of a membrane filter is the size of the pores. For sanitary bacteriological analyses of water, membrane filters with an average pore dimension of 0.7 microns are satisfactory. This value is derived from the filtration rate of distilled water on the basis of Poiseuille's law (cf. Lander and Zakovskiy, 1937, page 93).

As is evident from the earlier description of the structure of membrane filters, the dimensions of different pores may vary at times both towards greater as well as towards lesser size and for this reason it is important that bacteriologists have data on maximal pore size.

This index is obtained by measuring the pressure necessary for the passage of air through a wet filter (cf. Lander and Zakovskiy, op. cit., page 121).

Satisfactory direct counts of bacteria may be obtained with those membrane filters on whose surface is produced a regular spot by the passage of a dilution in distilled water whose suspended bodies do not settle during 24 hours and where there is no color on the reverse side of the filter and where the filtrate is transparent.

Membrane filters can be kept for a long time both in the dry state and in water without changing their properties essentially. They may be sterilized in boiling water and in an autoclave. The size of the pores decreases somewhat during these processes.

The filters must be elastic, able to be folded double without cracking.

Before use new filters should be boiled in water to remove air and extraction agents from them. During this process they must not curl up or change other properties. According to Taylor and others (1953), American filters which he tested, as distinct from the German ones, curled up following boiling in water and sterilization and as a consequence do not stick well to agar and other media, shrivel up a little and become brittle. For this reason it is recommended that they be sterilized with ethylene oxide (explosive) or ultraviolet radiation. (We find a reduction in visible count after ultraviolet irradiation).

Our membrane filters are very elastic, they can be handled and folded many times without damage but some batches show more or less marked buckling when boiled. The filters made in the US have a grid printed on them with each square corresponding to 0.01 of the filtration surface which makes a count of growing colonies much easier. (also reduces the visible count)

FILTRATION APPARATUS

There are a great many apparatus for filtering through

membrane filters. Description and pictures of those which are used in ultra-filtration may be found in a number of special manuals (Khanaer and Zikovskiy, 1937; Tovarnitskiy and Glukharev 1951, and others).

For examining water the following are used: Kolkwitz' apparatus proposed for separating plankton from water (Kazumov,1932 Figure 1); Seitz' apparatus of different dimensions, recommended for filtration through asbestos-cellulose membranes (cf.Pianova and Voroshilova, 1932, Figure 1); two Olikhov apparatus for studying plankton (cf. Kazumov, 1933, Figures 1 and 2). The handiest and simplest for microbiological studies of water is the S. S. Semenov apparatus and the very similar one made by Engineer Gol'dman. Its lower part has an area with a round opening closed with a porous layer of crushed glass or one cut out of punice, brick or other porous material (diameter 30 millimeters) and ending in a double tube: the outer tube is fitted with a spigot and connected to a vacuum pump: the inner tube communicates with the porous layer and serves to carry off the filtrate. The stopper which closes the bottle to receive the filtrate needs only one opening. The upper part is fitted with a metal or glass funnel holding up to one liter. The lower part has two wedge-shaped flanges, the upper part corresponds to the shape of the grove which assures air-tight coupling. Many laboratories maintained by our water companies are equipped with

special tables with a battery of filtration apparatus (6 to 10 pieces) supplied with gas and vacuum and a lead-off for the filtrate.

Judging from prospectuses this type of apparatus is widely used in Germany.

The most widespread in the US is Goetz' apparatus (Goetz, 1951, 1952) basically the same as that of S.S. Semenov. Its upper part is a cylindrical funnel of stainless metal while the lower part has a layer of "porous carbon" with a filtration surface of 9.6 square centimeters. There is no lead for pumping out air so the receptacle for the filtrate may be only Bunsen flasks. The presence of a rubber washer and the use of plastic in making some parts of the apparatus excludes the possibility of sterilizing it by heating. The apparatus is sterilized disassembled with formalin vapors produced by incomplete combustion of methyl alcohol in a chamber adjacent to the apparatus.

Goetz (1953) proposed a special filtration device, the concentrometer. Its principle feature is the fact that the membrane filter is placed vertically and not horizontally as usual. As filtration progresses the amount of suspended matter and bacteria increases from the top of the membrane filter toward the bottom (the suspended matter is deposited in the form of a wedge). This method of filtration is possible since a wet filter is impenetrable

to air at no less than 3 atmosphere pressure.

The membrane filters are rectangular (3x4 centimeters) and provided with a frame on which are marked the horizontal levels corresponding to the standard volumes of analysed water used in the US (from 0.1 to 100 millilitres). This permits the use of computation tables for the coli index (MPN - most probable number of Escherichia coli). After filtration the membrane filters become overgrown in the usual manner.

A very simple apparatus for direct bacteria count in water (microbial plankton) is the Pol'sh "pinchcock No.1" (Pol'sh, 1935). Most useful for this purpose in the modification by S.P.Bol'shov (Razumov, 1953). The lower part of the apparatus has an opening 10 millimeters in diameter closed with a porous layer instead of a metal sieve; the tube that conducts off the filtrate is double as in the S.S.Semenov apparatus which enables one to use any bottle as a filtrate receptable. The metal cup is replaced by a glass one which eliminates contamination with metal oxides, The two parts are joined as in the Pol'sh pinchcock (nut screw).

THE SCOPE OF USE OF MEMBRANE FILTERS

The membrane filters proposed by specialists in the field of physical and colloid chemistry were first used there as filtering material operating on the sieve principle whereby sorption phenomena, if there were any, were but slightly pronounced (cf.Ikander and

Zakovenky, 1937, pages 33, 44-76).

In hydrochemistry membrane filters are used for quantitative determinations of suspended matter (Lyubov and others, 1953, page 75).

For a qualitative and quantitative study of plankton, and nanoplankton in particular, membrane filters were first used by Kolkwitz (1924). We designate organisms separable from water by membrane filters as "membrane" plankton. For this purpose we use preliminary (plankton) or No.4 and 5 membrane filters (cf. table) and the Olikhov filtration apparatus. These preliminary membrane filters are employed for helminthological examinations of bodies of water (Vasil'kova, 1941).

We have certainly not exhausted the possibility for using membrane filters in various microbiological studies, especially in those cases where a small amount of microbes are found in comparatively large quantities of liquid media. The finepore membrane filters especially are finding use in virusology (cf. Tovarnitskiy and Glukharev, 1951).

In addition, membrane filters can produce filtrates free of bacteria, their filtrable forms and viruses (Requires special membrane filters for this). They may even be used on a more extensive scale for decontamination of water in private homes, hospitals, pharmacies, laboratories, etc. (cf. Reichenbach, 1930, for example).

In the field of general and sanitation microbiology membrane

filters were first used with great success, as later shown, here in the USSR. It was likewise the USSR which first proposed the use of membrane filters for studying the microflora of the air (Milyavskaya, 1947).

MICROSCOPIC STUDIES OF WATER WITH MEMBRANE FILTERS

There can be no doubt that it has been firmly established that the microbial population of the seas, oceans and continental waters is hundreds, thousands or even more times more numerous than we can conceive from methods based on the culturing of microbes (by counting colonies and determining the titer on all possible media). Realizing quite clearly the defects of the culture method in studying the microflora of water, many investigators have been seeking methods for direct, immediate examination. A very real difficulty in this field is presented by the search for a more simple and reliable method for concentrating relatively small numbers of bacteria from comparatively large quantities of water samples.

The most improved and simple method is that of filtering the quantity of water mentioned to the end, drying and fixing the microbes which have settled on the surface, staining this preparation with carbolerythrosin, washing the stain from the membrane filters with distilled water, a second drying, mounting in Canadian balsam and examining with a microscope.

Filters for studying the microflora of water must satisfy the following requirements (1) have an average pore diameter as small as possible (for example, 0.5 microns), (2)assure an even distribution of the suspended matter and bacteria over the filter surface, (3) be as thin as possible(something less than 0.1 millimeters is best) which limits the retention of stain by the filter body and increases contrast in preparations and (4) have a body that retains no foreign particles and, in particular, no microbial cells, spores, fungi or inorganic matter. Other requirements for membrane filters in general have been mentioned before.

These requirements may be met practically in the following manner:

The most practical membrane filters are those prepared according to Dianova and Verochilova's metho(1932) which lack a coarse network structure and have an ideally smooth, glassy surface on the "glass" side. Among factory-made filters the finest must be used (No.1 and 2). According to Goetz (1932) where membrane filters are 0.15 millimeters thick and have an average pore size of 0.7 microns bacteria penetrate to a depth of 10 microns, i.e., they are not distributed in one plane, which makes a count more difficult.

The body of the membrane filters must contain no mechanical contamination nor, in particular, any microbial cells or their spores. This type of inclusion may have a most unfavorable effect in studying

water with sparse microflora. Recently attention has been directed to this possible source of error by Kriss (1953), and his colleagues Rukina and Biryuzova (1952) have developed a method for making membrane filters which are free of this defect.

The problem of the contamination of membrane filters and its evaluation has been treated in detail in the pages of this magazine in the above-mentioned articles of Kriss, Rukina and Biryuzova as well as by Kuznetsov (1952) and Kuzumov (1952). The contamination of the membrane filter used may be easily evaluated by studying the edges which did not participate in the filtration of water while examining the stained material. Where there is a relatively small number of microbes in the water it is recommended that the quantity of water to be filtered be increased. It is of course impossible to use really contaminated batches of membrane filters without hurting the study being made.

As in counts on MPA (meat-peptone agar) not all clumps are to be regarded as colonies of bacteria so in this work not all particles that take the stain are to be considered as microbial cells, i. e., the work must be done by a rather experienced microscopist who is able to distinguish microbes from chance contamination.

Direct methods for counting microbes in soil and, in particular, in sea water have been subjected to very sharp but wholly unjustified criticism by Kalinenko (1953). Finding that Okhotsk Sea water showed

6-8 colonies in cultures on IPA while direct count showed up to 40,000 per milliliter, he assumed that the data obtained by the first method was more reliable than that obtained by the second. For other investigators (Kriss and Rukina, 1949) sea water is some spots far from shore produced not a single colony in cultures of 1-2 milliliters of water. If we accept the accuracy of such proportions it is logical to deduce that in these waters microbes play no practical role since they are almost totaly absent.

As a matter of fact, if for a single microbe of one cubic micron volume there is one milliliter or 10^{12} cubic microns of water it is difficult to expect that such concentration microbes would present the eleborate quantities of metabolic products or active concentration of antibiotics and enzymes which would be delectable by any methods whatsoever.

The great variance in results of quantitative calculations by direct count or culture on ordinary media are caused primarily by the presence in natural waters of numerous specific microflora which will not grow on these media. Both in fresh and sea water the direct method has discovered distinctive microorganisms which had been unknown up till then even though not infrequently exisiting in large quantities (cf.Razumov,1953; Kriss, 1949; Shturm, 1950). There is no basis for conjectures that microbial plankton include a very large number of dead cells.

We do not know of a single biocoenosis in which there would always be a large number of dead bodies for an unknown reason. The water from natural bodies cannot be equated with a fixing agent and for this reason dead cells rather quickly undergo decomposition as the result of autolysis as demonstrated by Lazarova (1953). It is possible to make a direct evaluation of the number of dead cells in water; they are usually found within the limits of 10-20 percent (Lazareva, 1953).

Finally, it is possible, despite Kalinenko's statement, to approach a determination of the qualitative make-up of this biocoenosis according to the morphological characteristics of the microbes and microchemical reactions (Gram staining technique, determining the storage of iron, calcium, sulfur, etc in the cells) as well as by using methods for microcultures on membrane filters and on special media (Razumov, 1953).

These preparations are used likewise for studying abiosestone (in computing the amount of ferric hydroxide in lake water-Kuznetsov, 1952, page 264) in studying mineral suspensions in rivers (Razumov, 1954, page 20).

The direct method for counting bacteria in water by using membrane filters has hardly attracted the attention of foreign microbiologists. Almost the only exception is the work of Zeltti (1935) which confirmed the main conclusions of Russian authors. During the past 2-3 years mention has been made of the possibility in principle of

using membrane filters in the field (Taylor and others, 1953) while only Jannasch (1953), basing his studies on Russian works, has published a modification of the direct bacteria count method using membrane filters and the results of using it in studying the microbial plankton in the Gulf of Naples. The features of the modification are as follows: the membrane filters are dried for 20 minutes at 70 degrees after water has passed through, the preparations are stained for 3-5 minutes with methylene blue (0.5 gram stain, 10 milliliter alcohol (96%) and the 100 milliliters of distilled water) until the stain is deep blue and the membrane filters are clarified. The average error in quantitative calculations has been found to be between 4 and 5 percent. Using a base-type stain, the author accepts the deep staining of the membrane filters and tries to increase contrast by using mixtures of various clarifying agents, for example, equal amounts of clove and immersion oil or paraffin oil and eugenol (Jannasch, 1954). Finally there is a short report (Richards and Krabee, 1954) on the possibility of examining microorganisms on membrane filters with a phase microscope.

METHODS FOR GROWING BACTERIA ON MEMBRANE FILTERS

Since 1932 in Russia and recently abroad great success has been had in using methods for concentration of water microbes on membrane filters with subsequent growth on various diagnostic media.

These methods were first proposed and given thorough study by Barsov (1932, 1933, 1947). He demonstrated that if the indicated amount of water is passed through membrane filters and placed in a Petri dish with NPA or Endo's medium, it is possible with ordinary culture systems to make a sanitation bacteriological study of water. As his observations have shown for a number of years, the number of colonies of a saprophytic microflora develops the same (or even somewhat more by the exclusion of damage to cells when the nutritive medium is poured in) as when this is done in the usual way. Coli indexes obtained through determination on membrane filters are the same as those from surface cultures of water on Endo's medium or by the titer method on standard liquid media. This approach to studying water and other liquid media seemed to posess a number of important advantages and for this reason soon gained the attention of Russian bacteriologists, hygienists and workers in the field of sanitations. In particular:

1. This method allows a considerable reduction in the needs of laboratories for bacteriological vessels, for the number and capacity of incubation, for culture media and materials and reagents which are necessary for their production.

2. There is a corresponding reduction in the amount of work for personnel occupied with milding analyses.

3. The whole system of sanitation bacteriological analysis of water may be shortened and its time reduced to 2 instead of 3-5 days as demanded by the standard method using fermentation tests.

4. As for the features of this method for determining the amount of various bacteria in water and, in particular, the coli bacilli group, it has the following advantages:

(a) The filter on separates the microbes from the water and thus excludes the possible unfavorable effect of bacteriophages, microbe antagonists, toxic substances in sewage, chlorine in drinking water, etc;

(b) Where there is a small amount of suspended matter and bacteria one filter may be used to concentrate large quantities of water (up to 1 liter and over) which is of great importance in analyzing artesian and purified tap water;

(c) Errors connected with the preparation of dilutions and encountered in pouring thick media are eliminated;

(d) The results of anlysis are expressed in the number of colonies (for example, the coli index) which is much more exact than determining titer;

(e) When analyzing water for the number of coli bacilli, pseudo fermentation titers caused by the multiplication of anaerobes are excluded;

(f) When Endo's medium is seeded the colonies of coli bacilli grow at 37-43 degrees for 24-18 hours and may be used for microscopic study of microbes and the isolation of cultures during further study;

(g) After drying and decontamination (by formalin, for example) the filters on which colonies have been growing may be kept as supplements to the records of analysis.

In 1939 in Russia by order of the People's Commissariats of Public Health and Communal Services RSFSR this method was introduced to replace the titer method and finally, 1950, it became the principal method in the first All-Union book of standards (GOST — 5281-50: Drinking water; methods for bacteriological analysis). The method of fermentation tests remains only as a substitute.

This method has found wide acceptance in various fields of applied microbiology in sanitation studies of water (Alfimov, 1954; Pozybay, 1933), of beverages (Gronval'd and Kanovskaya, 1945), of mineral waters (Lashchilina, 1946), for the detection of pathogenic microbes in water (Mats, 1941), in milk and milk products (Askolonov and Faybyshenko, 1940), in air (Milyavskaya, 1947) etc. A great number of works treating the details of this method and particular problems in its applications have been published in Gigiyena i sanitariya (Hygiene and Sanitation), Laboratornaya praktika (Laboratory Practice), Mikrobiologiya (Microbiology) and other publications since 1932.

Mention must be made of difficult cases of using membrane filters in studies of water.

Where there is a large amount of suspended matter and a

relative small number of bacteria the accumulation of suspended matter progresses more rapidly than does that of the bacteria.

The layer of suspended matter, by changing the conditions under which the colonies develop, may diminish the selective properties of the media used. To a certain degree this influence of the suspended matter may be limited by increasing the size of the membrane filter which, naturally, requires but a slight change in the construction of the filtration apparatus. This method is used both in Russia and abroad. Goetz and others (1951) propose for this case the use of larger membrane filters with 90-millimeter diameters.

For the same purpose we use filtration through 2 membrane filters, on top of a fine filter we place a larger pored No.5 or a "preliminary" membrane filter. When filtration is finished both filters are placed on a thick medium and the number of colonies grown are added together.

Potkov (1951) proposed a special modification of an apparatus for similar two-stage filtration. This method likewise does not solve the problem in case of an excess of very fine suspended matter as is characteristic of mountain streams and many rivers of Central Asia (Razumov, 1954).

Special difficulties are presented by the presence of plankton in the water. In this case the suggestion has been made to increase the selective quality of Endo's medium by introducing 0.1 percent phenol or to increase the concentration of fuchsin while the amount

of sodium sulfite remains unchanged (Razumov, 1947,1953).

To avoid the precipitation of ferric hydroxide from some mineral waters it is recommended that the ferrous oxide be stabilized by sodium citrate (Lashchilina, 1946).

As shown by Skoredumov (1933), bacterial colonies decidedly dehydrate a thick medium on which they are growing.

This phenomenon is even more pronounced in cultures on membrane filters: under large colonies there are clearly,visible pits which point out the difficulty in this case of a lateral subcurrent of liquid to the colonies, and in general the size of colonies on media with ordinary amounts of agar (1.5 - 2.0 percent) is somewhat reduced.

To speed up the growth of colonies on membrane filters Barsov (1937) proposed to decrease the amount of agar in media to 0.75 percent or even eliminate it entirely, by effecting the growth of cultures on beds of filter paper soaked in a nutritive medium. A number of similar absorbents of liquid media (sand, ground glass, etc.) were proposed by Dianova and Voroshilova (1943).

Membrane filters have opened up new and extensive possibilities in the field of accelerating bacteriological analyses of water.

Along this line Barsov (1937) suggested not only a decrease in the amount of agar in nutritive and diagnostic media but an increase in the amount of peptone in them, the introduction of

supplementary nutritive and growth substances in the form of meat extract, meat lysate and yeast autolysate, and an increase in the growing temperature to 41 degrees. This method succeeds in reducing the time for the appearance of colonies of coli bacilli visible to the naked eye from 24-18 to 10-12 hours. Strakhova proposed the introduction of vitamin B_1 into accelerated media (1948). Further mechanical reduction in the growing time leads to microscopic observation of primordial microcolonies. In this case the membrane filters are cultured for 5-8 hours and then removed from the culture medium and treated as preparations for direct bacteria count (Mazumov, 1933).

In this way Barsov (1937) succeeded in finding coli bacilli in water from the Moscow River after 3 hours of growth. The possibility of transplanting colonies grown on membrane filters to other media opens broad prospects not only in the field of sanitary bacteriological investigations but in studying the various processes connected with the metabolism of the most varied bacteria. M.F. Lazareva, at our suggestion, carried out simultaneous detection of a number of saprophytic bacteria and coli bacilli in one culture; the water was seeded on meat peptone agar and the colonies counted; then the membrane filters with the colonies were placed on a diagnostic medium. In the case of Endo's medium red colonies appeared within 2 hours, i.e., it is possible to detect bacteria capable of decomposing lactose with the formation of acids and aldehydes.

Diagnostic media usually contain different types of inhibitors of growth of outside bacteria (sulfin and other dyes, phenol, etc) which prolong the lag phase in the reproduction of bacteria. A more rapid transition to the phase of logarithmic growth may be obtained by growing the bacteria for 2-4 hours on accelerated media and completing the development on selective media.

With the works of Sander (1934) and Koeffling (1934-1935) as a basis, tests were made of the introduction of complex cyanides of copper, nickel, sodium nitroprusside and others into nutritive media.

As shown by our experiments, carried out with the collaboration of Mudretsova-Vies and Orlova, it is possible within a short time to establish the capacity for decomposing complex cyanides of copper and nickel by transplanting membrane filters with colonies grown on meat peptone agar to media with the proper supplements. In the latter case the nickel ions in the colonies were detected by "developing" the membrane filters with Chugayev's reagent (dimethylglyoxime). On meat peptone agar with 0.5 percent sodium nitroprusside, bacteria of the coli bacilli group form blue colonies. Gram-positive and many gram-negative bacteria do not have this capacity.

On nutritive media with a source of carbon in the form of iron citrate or potassium ferrocyanide blue colonies of citrate-assimilating bacteria grow. It is better, however, to introduce only iron citrate into the medium and remove the filters with colonies from the medium

and "develop" them by moistening them with a solution of potassium ferrocyanide (3 percent) and hydrochloric acid (5 percent).

Furthermore one may very quickly establish the capacity of bacteria growing on membrane filters for reducing nitrate into nitrites: the filter with the colonies is placed on agar with an addition of KNO_3, KI and soluble starch, kept at a temperature of 37-41 degrees for 15-30 minutes, removed from the medium and the medium is covered with hydrochloric acid (5 percent). The iodine taken from the potassium iodide by the nitrite produces blue spots under the proper colonies. The filter with the colonies of bacteria may be placed in sequence for a short period on a number of media and, using the appropriate macro-.and microreactions, a detailed description of the biochemical properties of the bacteria studied may thus be obtained. Diagnostic media for these analyses may be prepared ex tempore, starting with "unfed" agar, meat peptone agar, and others, and adding the required ingredients under sterile conditions. Membrane filters with colonies on them may be kept for days if dry, for example, in Petri dishes. The activity of bacteria in the colonies during this process will remain quite high.

With the correct density membrane filters are impenetrable for bacteria. Experiments with cultures of bacteria in tubes to one end of which membrane filters were attached with collodion and placed in flasks show the absence of any growth of bacteria through the filters. Certain mold fungi and actinomycetes capable of destroying

the substance of the filters do grow through the membranes. This property of membrane filters may be utilized in vivo studies of colonies of microbes, phyto- and zooplankton, and others (Razumov, 1953).

Membrane filters are irreplaceable for making field and expediation studies of bodies of water. Here the following opportunities are especially valuable: (1) doing without liquid nutritive media, (2) reducing the amount of equipment needed and (3) having a simple solution for the difficult problem of the incubator which has not yet been satisfactory either in Russia or abroad. Barsov (1932) proposed that in these cases one substitute metal (for example, aluminum) cans for the Petri dishes and grow the cultures by placing them in the pockets of a special vest, thus utilizing the heat of the body. We have available a portable laboratory which is adequate for all work in studying water in situ (selection of samples, processing them, obtaining answers and collecting material for use in the home laboratory to make the results of analysis more exact). Instructions for carrying out these operations have been published several times (Razumov, 1943, 1947, 1953). Using this method Kuznetsova and I made an extensive examination of sources of water under extremely difficult conditions in districts only recently liberated from occupation during the past war. In the US there are portable laboratories with a thermos for growing cultures on membrane filters(Goetz, 1953b). At present the method for growing cultures on membrane filters is undergoing intensive development in Germany where it was carried from the USSR during

the Great Fatherland War. The first report on it there was published in 1943 with mention of the work of the Russians who first had the idea of placing the membrane filters on nutritive media. Kruse (1949) calls this circumstance "tragic", presumably having in mind the unused opportunity for overcoming the extreme difficulties of German bacteriologists during the war. Work is being done therein accelerating the sanitary bacteriological analysis of water (Kruse, 1949), on developing a standard analysis, and on using the membrane filter method.

Methods based on the use of membrane filters have passed to the US from Germany where the former country sent a special commision immediately after the end of the war to study the achievements of Germany, particularly in this field. In the US the new methods for studying water and air have interested both military (Chemical Corps of the US Army) and civilian sanitation agencies and the committee to standardize methods for analysis of drinking water and sewage. In general, research in the US follows the line of using and improving the membrane filter methods developed in the USSR and described above. The culturing of membrane filters takes place on a pad of inert material (for example, thick filter paper), impregnated with a liquid nutritive medium. In order to shorten the lag phase, yeast autolysate and other stimulators of colony growth are introduced into the nutritive media which are more concentrated than usual. For the same purpose there is a short perliminary culturing of the membrane filters (2-3 hours) on complete media after which the filters are moved to a pad im-

pregnated with selective or diagnostic media where the culture completes its growth. There are pads which are soaked in advance with the proper media and then dried and sterilized. Before being used they are moistened in a sterile Petri dish with 2 milliliters of water. There are also more complex pads consisting of three layers: an upper impregnated with a medium to speed up the frowth of colonies, and a lower impregnated with a selective medium which may penetrate through the middle layer after the lag phase has elapsed (for example, in 2 hours). The details of membrane filter methods developed in the US may be found in the publication of Goetz (1951, 1952, 1953a), Bush (1953) and others.

In England work with membrane filters has been limited to adopting methods worked out in the US and to testing their suitability for established practice of sanitary bacteriological analysis of water in that country (Taylor and others, 1953).

CONCLUSIONS

The preceding analysis and the far from complete list of Russian works on various problems in the use of membrane filters, primarily in the field of microbiological analysis of water, show that much work has been done in the Societ Union on this question and that in this field we have been more than 20 years ahead of foreign countries. This in turn imposes a great and responsible obligation to keep in first place. While many foreign countries are

now working intensively on the adoption and improvement of the new methods, Russia has already been using them extensively to study the microflora of water, milk, beverages, milk, etc.

We know that the value of the research methods is determined not only by practical considerations (simplicity, rapidity, low cost, accuracy) but by the scientific achievements made possible through their use.

In this connection, two principal directions in research using membrane filters have already given tangible results.

1. The study of microbial plankton has revealed a more probable amount of microbes in waters, an amount more in keeping with their supposed and true role. In continental waters and seas we have found a number of new microorganisms not growing on ordinary media.

2. The method for culturing microbes on membrane filters by transfering them to various media allows for a complete study of the biochemical properties of microbes and their interrelationships without separation into isolated cultures and for a more rapid and more simple fulfillment of a number of practical jobs. The possibilities for including fluorescent analysis, chromatography, phase microscopy and others have not been sufficiently utilized.

An invariable condition for progress in this direction is better production quality from our Experimental Membrane Filter Factory, a standardization of production, an increase in the variety

of membrane filters designed for various purposes, and a search for similar new filtering materials which do not have the few negative properties of membrane filters made from nitrocellulose. It is to be hoped that we will achieve great success in this direction.

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